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PRINCIPAL INVESTIGATOR: Dr. Michael Hilinski

CONTRACTING ORGANIZATION: Rector & Visitors of the University of Virginia
Charlottesville, VA 22903-4833

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Michael Hilinski

E-Mail: mh6cu@virginia.edu

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Rector & Visitors of the
University of Virginia
1001 N Emmet St.
Charlottesville VA 22903-4833

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14. ABSTRACT
RSK (p90 Ribosomal S6 kinase) is critical for breast cancer proliferation and thus a promising target for therapeutic intervention. A highly specific inhibitor of RSK, called SL0101, was previously discovered but found to possess poor biological stability and potency. The purpose of this project is to identify a drug for breast cancer based on SL0101 that works by inhibiting RSK, by designing and chemically synthesizing analogues of SL0101 that improve on its biological stability and potency and ultimately evaluating them in a living human breast tissue model for anticancer activity. The major findings in this year of funding are the discovery of analogues that are more biologically stable than SL0101 but retain its anticancer activity, the solution of an X-Ray crystal structure of SL0101 in complex with RSK2, and the use of this crystal structure in the discovery of an analogue that in preliminary experiments demonstrates improved potency over SL0101. These discoveries represent significant steps toward the goal of identifying an analogue of SL0101 that could be used as a drug for breast cancer.

15. SUBJECT TERMS
ast cancer, p90 Ribosomal S6 kinase, RSK, kinase inhibitor, medicinal chemistry, cell permeability

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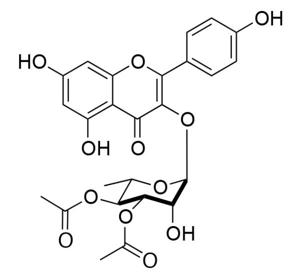
Introduction

The p90 ribosomal S6 kinase (RSK) family of serine/threonine protein kinases, comprising at least four isoforms (RSKs 1-4), has been shown to be critical for breast cancer cell proliferation (*1–3*). In 2005 Dr. Lannigan reported the first specific inhibitor of RSK, SL0101 (**1**, Figure 1) (*3*). SL0101 inhibits RSK in both the breast cancer cell line MCF7 and the normal breast cell line MCF-10A, but only inhibits the proliferation of the breast cancer cell line (*1–3*). This indicates that breast cancer cells have become dependent on RSK and thus identifies RSK as a potential new target for cancer therapeutics. SL0101, given its exquisite specificity for RSK, is an attractive lead compound for medicinal chemistry efforts aimed at discovering a breast cancer drug that acts by inhibiting RSK. However, SL0101 itself is not suitable for further development as a drug for two reasons. First, we have determined that SL0101 has a very short biological half-life in mice (0.4 h at 2.5 mg/kg IP). In order to develop a drug this half-life must be improved so that the drug persists in the patient long enough to act on RSK. Second, SL0101 is a potent RSK inhibitor (IC_{50} ~ 0.5 μM), but a much less potent inhibitor of the proliferation of MCF7 breast cancer cells (EC_{50} = 50 μM), suggesting that it does not readily pass through the cell membrane (*3*). In order to develop a drug, the potency against MCF7 cells must be improved. The scope of this project is to design and chemically synthesize analogues of SL0101, with the assistance of a computational model of SL0101 bound to RSK, that improve on these deficiencies and to evaluate them both in vitro and in vivo with the goal of identifying a new breast cancer drug that acts by inhibiting RSK. If warranted, the best analogues will be evaluated in our new living human breast tissue model (*4*) to gain insights in to the role of RSK in breast cancer that might not be gleaned from in vitro and cell-based assays.



SL0101 (**1**)

Fig 1. SL0101, a highly specific inhibitor of p90 ribosomal S6 kinase (RSK)

Keywords

Breast cancer, TNBC, kinase, SL0101, RSK, p90RSK, small molecule, MCF-7, MCF-10A

Overall Project Summary

In the previous years of the project (Years 1 & 2) both the in vitro biological stability and ex vivo potency of SL0101 analogues were improved. The biological stability was improved by modifying the substitution on sugar portion of SL0101 to replace the biologically labile acetates at the 3' and 4' positions of the carbohydrate ring with relatively biologically inert carbamates (*5*). Importantly, these new analogues retained specificity for RSK. As expected, this type of structural modification did not improve substantially the potency of the compounds for inhibition of RSK kinase activity (i.e. in vitro potency) nor for inhibition of cancer cell proliferation (i.e. ex vivo potency). In Year 2, the ex vivo potency of SL0101 analogues was improved by designing analogues with the aim of increasing cell permeability. This work was aided by a crystal structure of SL0101 bound to the N-terminal kinase domain of RSK2 obtained in Year 1 (*6*). The best analogues discovered in year two were on the order of 10-fold more potent than SL0101 at inhibiting the proliferation of breast cancer cells. One potential explanation for this considerable increase in potency is that the new analogues have decreased specificity for RSK, which needed to be addressed before moving forward with the new analogues. This question became a focus of work performed in Year 3, along with combining the features of both the more stable and more potent analogues into a single analogue that could be carried into the proposed animal experiments and other experiments proposed for Year 3. What follows is a detailed description of the results obtained relevant to the Statement of Work for Year 3.

Task 5: In collaboration with Michelle Rudek-Renaut, evaluate the biological half-life of 1-3 analogs in CB17 SCID mice for both subcutaneous and intraperitoneal routes. (Timeframe: months 1-6 and 21-27)

Task 6: Evaluation of up to 3 analogs in our breastoid model. This will require the use of 15 human tissue samples, which we will collect under an approved IRB protocol that protects patient identity. (Timeframe: months 1-6 and 28-36)

These tasks are not scientifically justified until an analogue with suitable potency, specificity for RSK, and in vitro biological half-life can be identified, and were therefore delayed until a suitable analogue could be chosen. As reported previously, during Year 2 a initial set of SL0101 analogues bearing an ether functional group at the C2'' position of the rhamnose were synthesized (Figure 2). These analogues the same to slightly worse than SL0101 at inhibiting RSK2 kinase activity (Figure 2). However, the initial hypothesis was that due to increased lipophilicity these analogues would be better able to penetrate the cell membrane and would exhibit higher potency in ex vivo (cellular) assays. Initial cell proliferation data for a subset of analogues were presented in the previous year's report. In Year 3, the ability of all new C2'' ether analogues to inhibit the proliferation of the MCF7 breast cancer cell line was determined (Table 1). A general trend was observed wherein the IC_{50} decreased with increasing lipophilicity of the C2'' ether substituent. Notably, all of the new C2'' ether analogues were more potent than SL0101 in this assay. Since analogues **2-5** did not exhibit increased potency for inhibition of RSK, these data are consistent with the hypothesis that increased lipophilicity would enhance the ability of the analogues to penetrate the cell membrane.

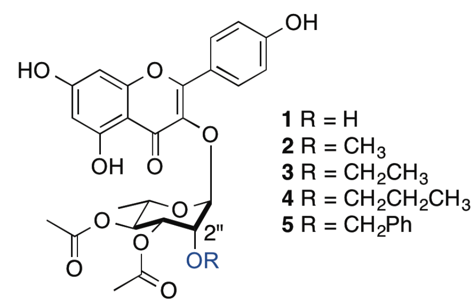


Fig 2. C2''-ether analogues of SL0101

An alternative explanation for the increased MCF7 cell proliferation assay potency of these compounds in light of their modest RSK inhibitory activity is that they are no longer specific for RSK. If true, this could be highly detrimental to their potential as breast cancer drugs due to off-target biological activity. Before moving forward with additional analogues based on this scaffold, we needed to first determine whether RSK specificity was maintained. Initially, determined the ability of the most potent analogues, **3-5**, to inhibit the proliferation of the normal breast cell line MCF-10A (Figure 3). It has previously been reported that the analogues that are specific for RSK exhibit a greatly reduced potency to inhibit the growth of non-transformed cell lines (*3*). Analogues **3-5** did not inhibit the growth of the MCF-10A cell line at their respective IC_{50}'s for inhibition of MCF7 proliferation, providing evidence that they retained specificity for RSK. As a further measure of RSK specificity, we looked at a downstream marker of RSK inhibition in MCF7 cells. Figure 4a shows part of the signaling cascade that regulates eEF2 phosphorylation. RSK negatively regulates eEF2 kinase. As a consequence of inhibition of cellular RSK, peEF2 levels increase. Upon treatment of MCF7 cells with SL0101 or analogues **2-4** at 100 μM concentration a substantial increase in peEF2 levels were observed by Western blotting (Figure 4b). At lower concentrations, the ability of the analogues to inhibit RSK in cells was consistent with their relative ability to inhibit cancer cell proliferation. These data further support the conclusion that in this set of analogues, specificity for RSK is maintained. Further experiments to determine the specificity of these analogues against a larger panel of kinases are ongoing.

Analogue	RSK2 *in vitro* IC_{50} [μM]
1	0.877 (0.629-1.223)
2	0.601 (0.291-1.239)
3	3.939 (2.419-6.416) *
4	13.84 (7.549-25.39) *
5	38.17 (27.04-50.24) *

Table 1. Potency of analogues in the in vitro kinase assay. IC_{50} is concentration needed for 50% inhibition; the 95% CI is shown in parentheses; n=3 in triplicate; * $p < 0.05$

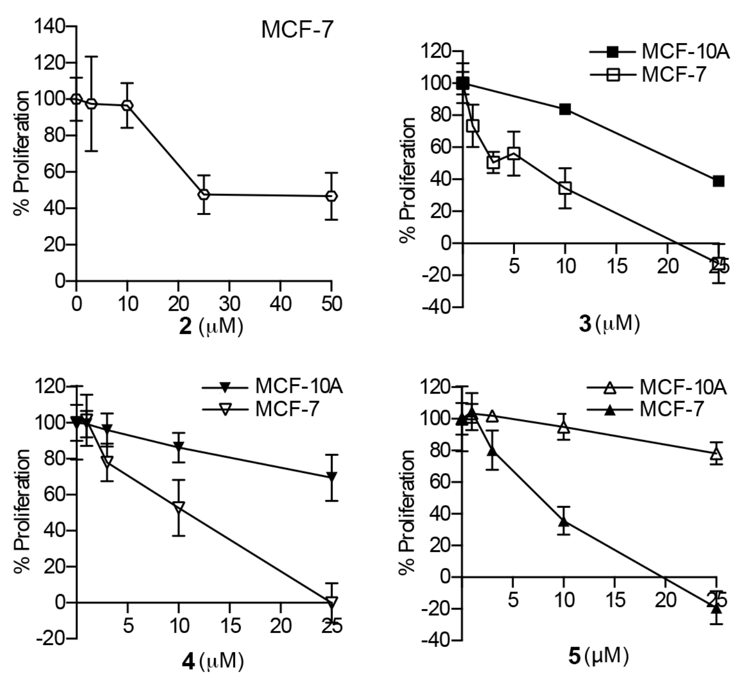


Fig. 3. Analogues **3**-**5** selectively inhibit MCF-7 over MCF-10A cell proliferation. MCF-7 or MCF-10A cells were treated with vehicle or the indicated concentration of analogues **2**-**5**. % Proliferation shown is normalized to treatment with vehicle.

We had previously determined that a SL0101 analogue lacking acetates on the 3'' and 4'' positions of the rhamnose moiety (i.e. a 3''-OH, 4''-OH analogue) lacked the ability to inhibit the proliferation of MCF-7 cells. We hypothesized that this was due to poor cell permeability. Given that the new 2''-ether analogues appeared to enter cells more readily, we sought to determine whether the 3'' and 4'' acetates were still necessary for potent activity in cell assays. The potential advantage of an analogue lacking the acetates would be increased biological stability, since we had previously determined that the acetates are a substantial metabolic liability (*5*). To test this hypothesis, I prepared an analogue **6** bearing a benzyl ether at the 2'' positon and hydroxyl groups at the 3'' and 4'' positions of the rhamnose (Scheme 1). Unfortunately, this analogue was roughly 4-fold less potent than the corresponding acetate analogue **5** at inhibiting the proliferation of MCF7 cells. Based on this result, this particular line of research was de-emphasized.

Having gathered evidence that the analogues **2-4** retained specificity for RSK, we decided to select a single analogue to serve as a platform for the development of a new RSK analogue that combines structural features that improve potency with structural features that improve biological stability. This analogue would then be suitable for in vivo work if potency and selectivity for RSK were maintained. We determined that analogue **3** exhibited the best combination of RSK inhibitory potency and cell proliferation potency, and therefore endeavored to synthesize an analogue incorporating both an ethyl ether at the 2' position and *n*-propyl carbamates at the 3' and 4' positions, which we had previously determined to confer the ideal combination of potency and biological stability. In order to provide enough material for extensive in vitro and later in vivo studies, we targeted the synthesis of 50 mg each of both the new carbamate analogue **16** and analogue **3** (to be used as a comparison). To achieve the most efficient synthesis of each analogue we devised a synthetic route that could produce both analogues (Scheme 2). Starting from known rhamnose derivative **7**, alkylation with iodoethane followed by deprotection under acidic conditions gave diol **9**. Acetylation followed by treatment of the resulting diacetate with bromine provide glycosyl bromide **11**, which was coupled to flavone derivative **12**. The resulting advanced intermediate **13** was used to prepare analogue **3**. In addition, new carbamate analogue **16** could be prepared from intermediate **13**. Hydrolysis of the 3'

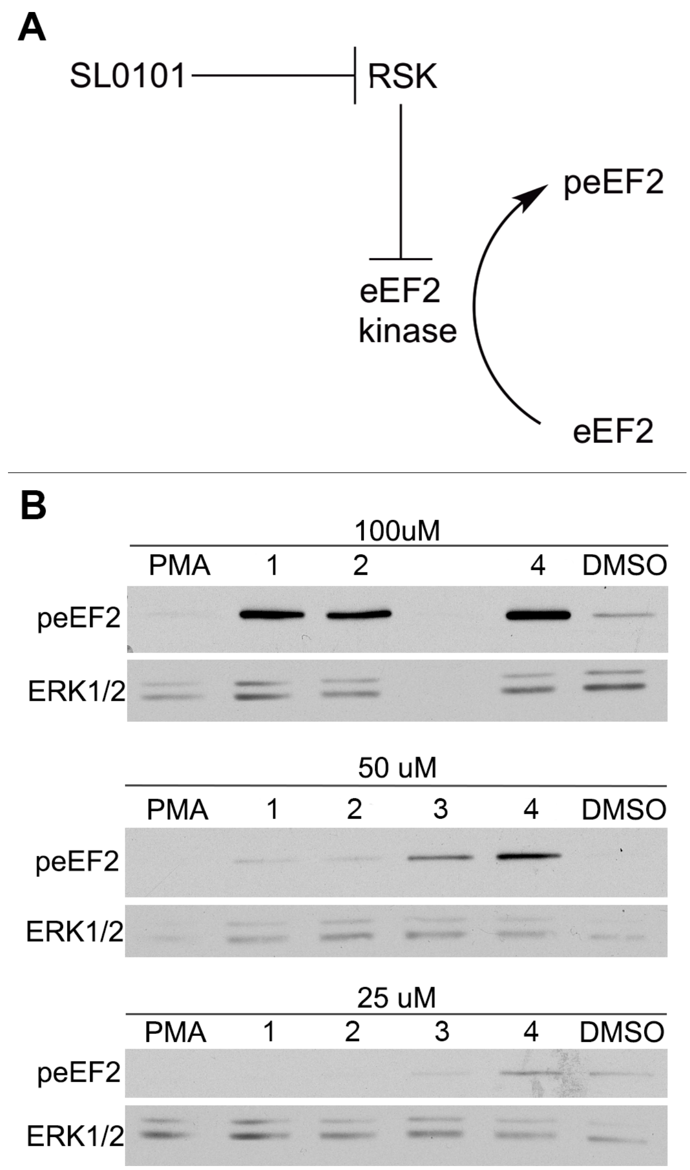
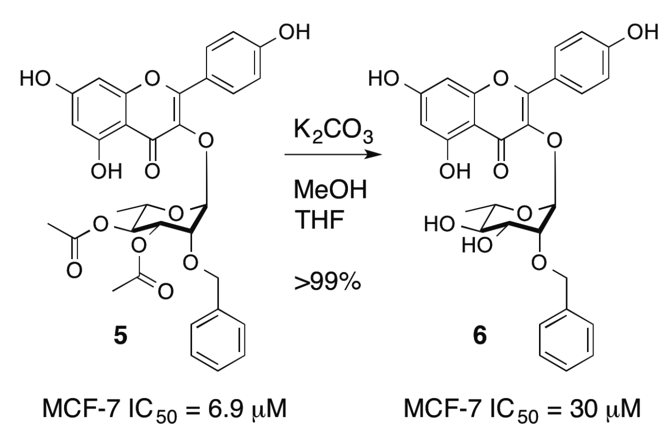


Fig. 4. (A) RSK regulates eEF2 kinase and therefore inhibition of RSK leads to an increase in peEF2 levels. (B) MCF-7 cells were treated with the indicated concentration of analogues **1**-**4** and after lysis cellular peEF2 levels were observed by Western blotting.



Scheme 1. A diol analogue bearing a C2''- benzyl ether.

and 4' acetates was followed by formation of the carbamate **15**. The analogue synthesis was completed after hydrogenolysis of the benzyl protecting groups to provide **16**. Analogue **16** was tested in the in vitro kinase assay for its ability to inhibit RSK2 activity, and was determined to be equipotent to the corresponding diacetyl analogue **3**. Additionally, the ability of new analogue **16** to inhibit the proliferation of both MCF-7 and MCF-10A cells was evaluated (Figure 5). The analogue was approximately 15-fold more potent than SL0101 at inhibiting the growth of the cancer cell line. Additionally, it did not inhibit the growth of the normal cell line at its IC_{50} for MCF-7 proliferation, indicating that it is specific for RSK. Further evaluation of its specificity against a larger panel of kinases is in progress.

It is expected that analogue **16** will exhibit the same biological stability of carbamate analogues we previously reported (*5*). Evaluation of its in vitro biological stability is in progress. Coupled with its substantially improved potency in cell-based assays over SL0101, analogue **16** exhibits the properties desired of an analogue to be used for in vivo studies. Thus we anticipate that we will use analogue **16** in additional experiments to complete the statement of work.

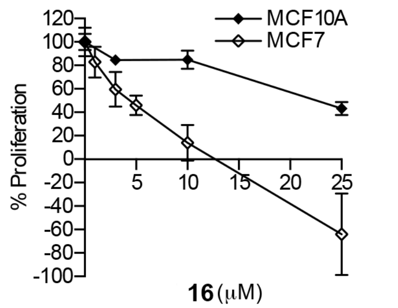
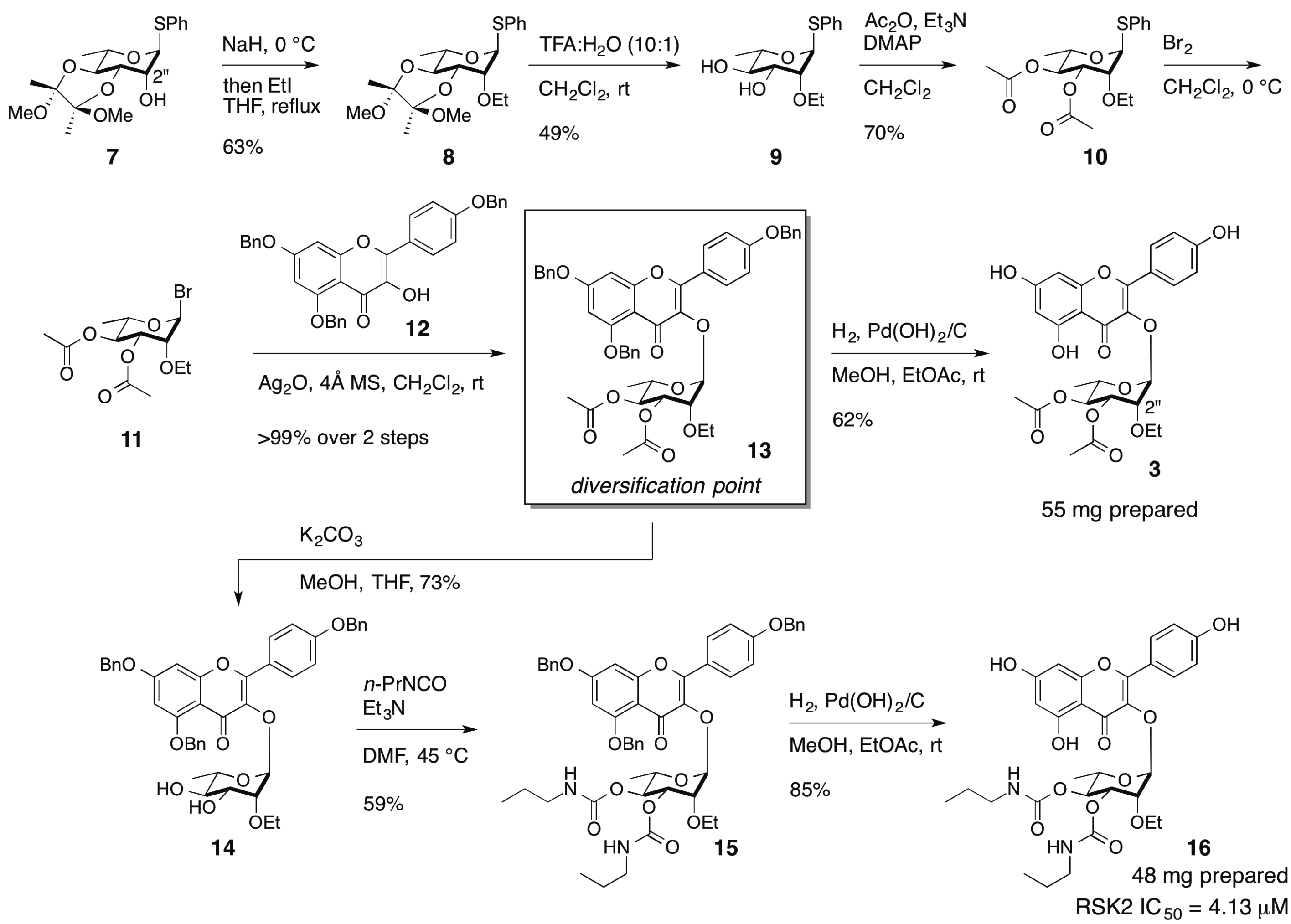


Fig. 5. Analogue **16** selectively inhibits MCF-7 over MCF-10A cell proliferation. MCF-7 or MCF-10A cells were treated with vehicle or the indicated concentration of analogue **16**. % Proliferation shown is normalized to treatment with vehicle.



Scheme 2. Resynthesis of analogue **3** and synthesis of a new analogue **16** combining a 2''-ether modification with 3'' and4'' carbamate modifications, previously shown to confer improved biological stability.

Training Plan:

Task 3: Regularly attend cancer and chemistry seminars. (Timeframe: 1-36 months)

I regularly attended seminars run by the University of Virginia Cancer Center and in the University of Virginia Chemistry Department during Year 3 of the project.

Task 6: Attend international meeting held in the US and BCRP Era of Hope meeting to present and discuss work. (Timeframe: 25-36 months)

The BCRP Era of Hope meeting was not held.

Key Research Accomplishments

- The discovery of an analogue of the RSK inhibitor SL0101 that is approximately 15 times more potent than SL0101 at inhibiting the growth of breast cancer cells, that retains selectivity for RSK, and that is predicted to exhibit improved biological stability as compared to SL0101. This analogue is a candidate for in vivo evaluation.

Conclusion

SL0101 is a promising lead compound for medicinal chemistry efforts to develop a breast cancer drug that works by targeting RSK. However it suffers from poor biological stability and potency, making it unsuitable for use as a drug. The discovery of analogues of SL0101 that are more biologically stable and that are more potent in cell-based assays as described in this report is thus highly significant as they overcome these deficiencies and therefore could find use as breast cancer drugs. In particular analogue **16** is both more potent and predicted to be more biologically stable than the parent compound Sl0101. Overall, the work accomplished in this year of funding moves the science of RSK inhibitors closer to the goal of a breast cancer drug that works by inhibiting RSK. Future plans to achieve the goals and objectives of the project include the evaluation of analogue **16** in in vivo models.

Publications, Abstracts, and Presentations

Nothing to report.

Inventions, Patents, and Licenses

Nothing to report.

Reportable Outcomes

Nothing to report.

Other Achievements

Nothing to report.

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Appendices

none